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14. ABSTRACT Introduction: The complications of radiation dermatitis and fibrosis on breast cancer therapy are well established. To date, the only proven approaches to reducing radiation toxicity are to decrease the treatment dose or field-size. Altering these has the potential of reducing treatment efficacy and is therefore not advised. We proposed that drugs aimed at the suppression of cytokines involved in the initiation and perpetuation of radiation-induced inflammation would be beneficial in preventing normal tissue toxicity after radiation therapy. Of particular interest were inhibitors of tumor necrosis factor-alpha ± (TNFalpha±), interleukin 1 (IL-1) and transforming growth factor-beta (TGFbeta)Body: We examined a number of agents to suppress generally and specifically cytokines be believed affect the severity and progression of radiation fibrosis in women after breast irradiation. Importantly, IL-1 related interventions consistently reduced cutaneous fibrosis after irradiation. We tested thin in mice deficient in the IL-1 receptor 1, in mice treated with IL-1Ra the natural soluble IL-1 receptor blocker, and in mice treated with non-specific drugs that reduce IL-1 expression including COX2 inhibitors. All interventions greatly reduced the rate of fibrosis development and the severity of the fibrosis. A manuscript describing the most important experiments has recently been accepted for publication in the Radiation Research Journal and is attached in appendix I. Key Research Accomplishments: We have identified 4 agents that should reduce radiation fibrosis in women treated for breast cancer. One, curcumin is in development for national clinical testing. Others, including IL-1Ra, are being evaluated as radiation protectors in case of bioterror.

15. SUBJECT TERMS
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INTRODUCTION:

Complications of radiation dermatitis and fibrosis of the breast are a well established and seemingly unavoidable side effect. Breast cancer is among the few remaining oncological diseases in which high dose radiation exposure of the skin cannot be prevented due to the oncological anatomy of this disease. To date the only proven approaches to reducing radiation toxicity are to decrease the treatment dose or field size. Altering these has the potential of reducing treatment efficacy and is therefore not advised. We proposed that drugs aimed at suppression of cytokines involved in the initiation and perpetuation of radiation induced inflammation would be beneficial in preventing normal tissue toxicity after radiation therapy. Of particular interest were inhibitors of the IL-1 signal cascade and of the TGFbeta signal cascade. The impact of powerful inflammatory factors like TNF was also of high priority. Equally important is not reducing the beneficial effects of radiation by concurrently protecting the tumor. The aim of this grant was to measure the therapeutic gain of anti-cytokine agents that might both reduce normal tissue consequences and augment (or at least not diminish) tumor response.

BODY:

Hypothesis/Aim 1:
Inhibition of the IL-1 signal pathway will reduce cutaneous toxicity after irradiation. Reduction of IL-1 signaling is not expected to alter radiation related tumor reproductive inactivation.

We examined the IL-1 signal pathway with two experimental methods. First delivery of Kineret (IL-1Ra) and then when that ran into trouble, double checked the hypothesis using IL-1R1 knockout mice.

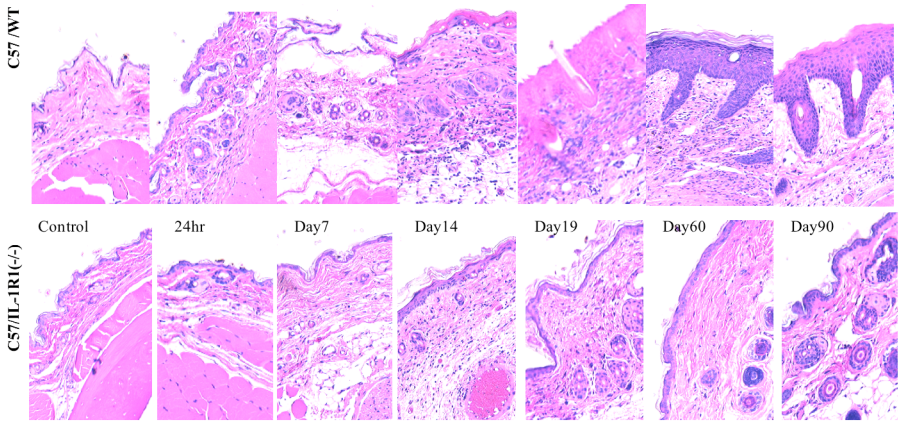
Briefly, Kineret is also known as IL-1Ra, a competitive inhibitor of IL-1alpha and IL-1beta for binding to IL-1R1. IL-1R1 is the receptor that transmits the signal when IL-1 is bound. Thus the IL-1R1 knockout mice have complete blockade of IL-1 signaling and the IL-1Ra treated mice have only a partial inhibition. In our experiments, we found that Kineret had benefit as a radiation protector, but required two weeks of daily high dose treatments in mice. Kineret is an antibody against the human epitope of IL-1. We believe that it did not perform well in part due to species differences between human and mouse and highlights the problem of using anti-human antibodies in mice. Based on this observation we made two changes in experimental design. First we replaced Embril in the experiments defined under hypothesis 3. This is because Embril like Kineret is an antibody, in this case directed against human TNF. Second, to answer the question posed by hypothesis 1 we used IL1R1 knockout (ko) mice. As noted above, this served as a true test of Hypothesis 1.

As predicted by the Hypothesis 1, the IL-1R1 ko mice had hugely reduced cutaneous toxicity that manifest both early (first few weeks) with reduced inflammatory changes and late (first few months) with reduced fibrosis. In this case, the complete IL-1 signal

pathway blockade present in the IL-1R1 ko mice produced pronounced effects in the mice (see figure). We believe the discovery that the IL-1 signal can powerfully modify toxicity of radiation to the skin, to be a paradigm shift. Based on this discovery we have a target that appears to be able to both prevent and treat radiation toxicity to the skin. A manuscript supported in part by the DAMD17-03-1-0732 research summarized in this report is attached in the appendix. Together with the data presented below, it demonstrates quite conclusively the role that IL-1 and the signal pathway play in the pathogenesis of radiation dermatitis.

Results

Figure 1.The histopathological progression of fibrosis supports the role of IL-1 signal effects after radiation, 30Gy (H&E [redacted]



Acute dermatitis seen at day 14 was markedly reduced in the IL-1R1 -/- compared with control C57 mice. Like wise, at 90 days IL-1R1 -/- mice had far less hyperkeratosis and had much less permanent depilation compared with the control mice.

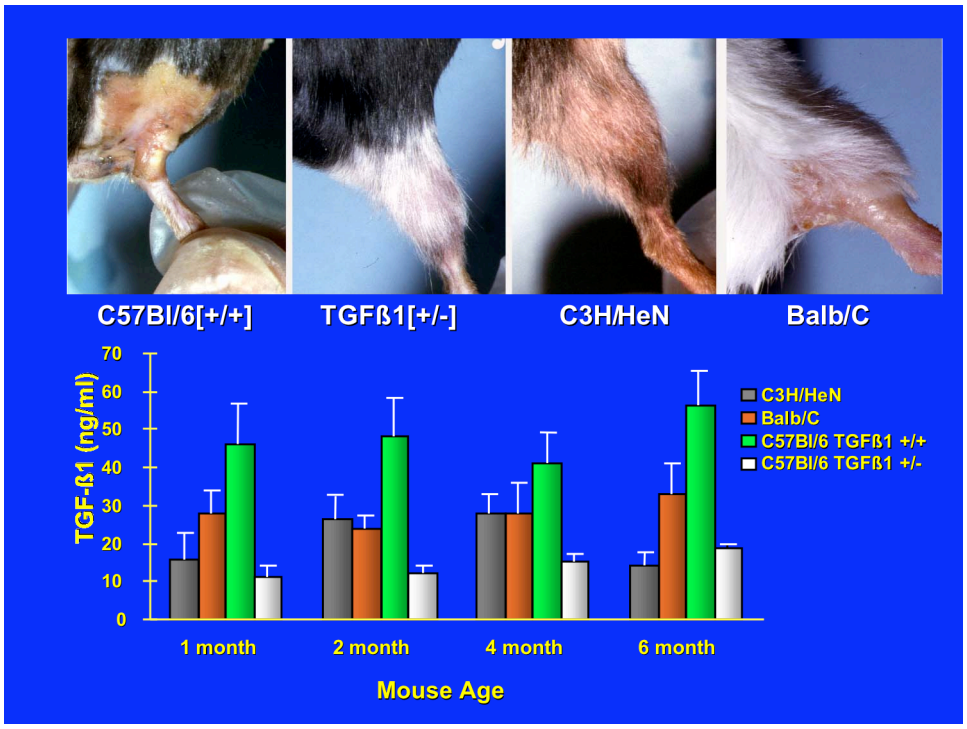
Hypothesis 2:

Inhibition of the TGFbeta pathway will reduce fibrovascular toxicity of cutaneous radiation

There are a number of TGFbeta binding proteins. We had originally hoped to employ decorin, a naturally occurring circulating TGFbeta binding protein. Decorin was not available. Based on the success experienced in Hypothesis 1 using knockout animals, we instead employed animals that are naturally deficiency in TGFbeta1 cytokine production. In this case TGFbeta1 heterozygous knockout mice were used. As can be seen from the data below, C3H mice are naturally low in TGFbeta throughout their life. C3H/HeN mice also have far lower levels of late fibrovascular complications after irradiation than most other strains. C57Bl6 mice have very severe fibrosis after irradiation. They have naturally high circulating levels of TGFbeta during their whole lives. Balb/C have intermediate levels of fibrosis and similarly intermediate levels of TGFbeta1 in the circulation. The heterozygous knock-out TGFbeta mice in a C57 background had low circulating levels of TGFbeta1 and like the C3H low levels of fibrosis after irradiation. The results provide a clear correlation and likely an explanation for the variability seen clinically in fibrovascular complications seen after irradiation between patients. The results also imply

that interventions that alter natural TGFbeta expression will prevent toxicity in sensitive individuals as it did in the C57 mice.

Figure 2: Role of natural production of TGFbeta1 on severity of fibrovascular toxicity after irradiation. As can be seen in the bar graph, animals that are not irradiated but allowed to age have levels of circulating TGFbeta1 that are strain dependent. C3H/HeN mice had the lowest levels of TGFbeta1 and the lowest sensitivity to fibrosis. The C57 had the highest circulating TGFbeta1 and worst fibrosis after irradiation. Balb/C were intermediate. If the TGFbeta1 site is made heterozygous in the C57Bl/6 mice, the level of circulating TGFbeta1 decreases and so does their propensity for fibrosis.



Hypothesis 3:
<u>Inhibition of powerful inflammatory factors, like TNF will reduce fibrovascular toxicity of cutaneous radiation</u>
Armed with the discoveries found in Hypothesis 1 and Hypothesis 2 we examined several powerfully anti-inflammatory agents that we expected would decrease IL-1 signals and TGFbeta signals. Most of these anticytokine agents also directly or indirectly alter TNF expression. Embril was abandoned based on the problems previous experienced with Kineret as explained in the discussion under Hypothesis 1. We used instead other promising agents: EsA, curcumin, and the COX2 inhibitor Celebrex®. We confirmed that each did decrease the levels of IL-1, TNF, and TGFbeta after irradiation. As shown below we were also rewarded with significant cutaneous protection against high dose radiation exposure. Some of the results were very impressive.

We then also examined these three agent for their effects on murine mammary cancers. The expectation being that decrease of the inflammation in tumors would logically lower

the growth factors that are seen in inflammation and prevent any tumor protection. This is critical if we are to have a therapeutic gain justifying the use of radiation protectors during curative cancer treatment. Here again we were rewarded. As shone for example in Figure 3, curcumin at 50 or 100 mg/kg/day given for five days before or 5 days after or 5 days before and5 days after irradiation all substantially reduced acute skin reactions seen 2 to 3 weeks after irradiation. Curcumin reduced TGFbeta1 and decreased TNF as shown in Figure 4. Finally, despite the protection of normal tissue the tumor growth delay was improved not reduced for murine mammary cancers (MCa35) compared to radiation alone (Figure 5). To demonstrate that the effect was true, we studied other drugs. For example, EsA powerfully reduced cutaneous toxicity, as shown in figure 6. COX2 inhibitor Celebrex® had lower benefit and less effect on the IL-1 suppresion compared to EsA at the doses chosen. As with cucumin, EsA and Celebrex (Figure 7) also benefited radiation response with a longer tumor growth delay. These results are exciting and though apparently contradictory, are easily explained by Hypothesis 3.

Fig 3. Curcumin protect the skin soft tissue from sever early dermatitis

A **B** **C**

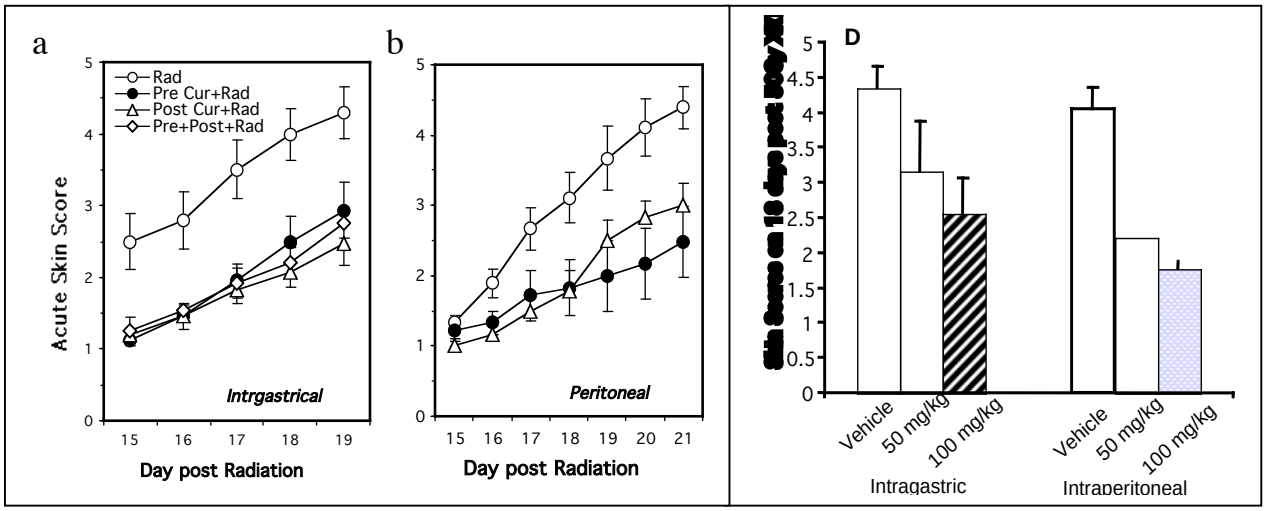


Figure 4. To determine the underlying molecular mechanism, RNase protection assay was used. This Figure shows that on day 21 (16 days after the last curcumin treatment), the IR induced **cytokines** (IL12 p35, IL12 p40, IL1 α, IL1 β, IL1 Ra, IL8 and IL6) in both skin and muscle were still greatly inhibited by curcumin. The sentinel cytokines from Hypothesis 1 and 2, TNF and TGFbeta, measured in cutaneous tissues are shown in the bar graph.

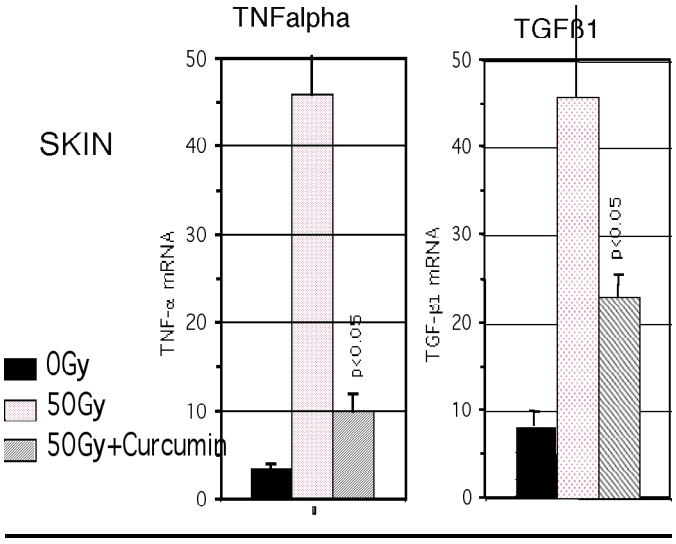


Figure 5. Tumor growth curves for control, curcumin (cur), irradiation (IR) and combined treatment are shown. Unexpectedly there was a moderate growth delay from curcumin alone. Perhaps because tumors have inflammation that includes growth factors and those are reduced by the anticytokine effects of curcumin. Raiation (10 Gy) produced approximately a 8-10 day growth delay. Adding curcumin added an additional 2 to 3 day growth delay indicating that the skin protection was complemented by a concurrent tumor sensitization.

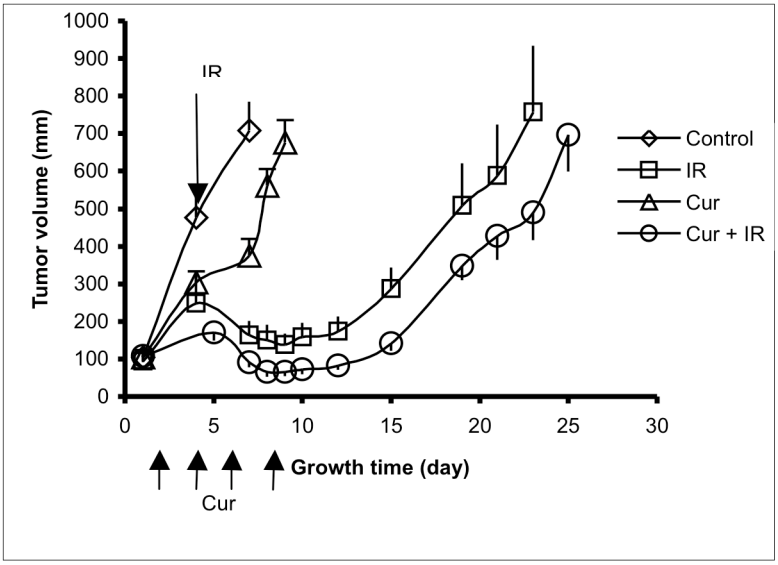
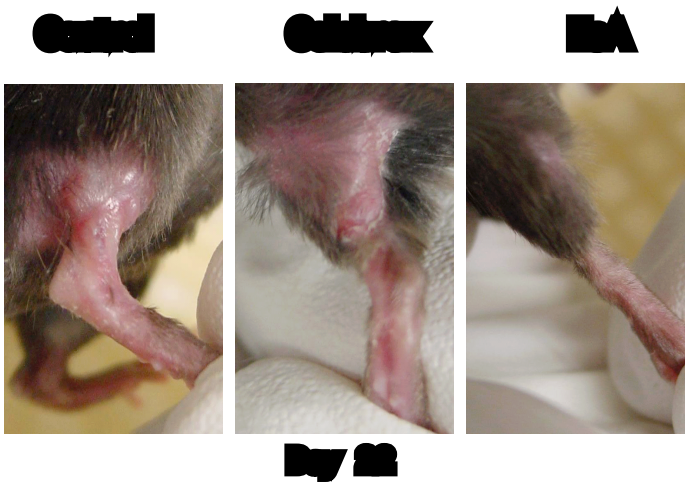


Figure 6. The hypotheses being tested predict a benefit to COX2 inhibitor Celebrex, and a powerful mult-mechanism anti-inflammatory agent, EsA. The EsA in particular almost completely prevented severe toxicity as shown below. C57Bl/6 mice legs treated with 30 Gy irradiation had desquamation and ulceration with complete epillation. Animals receiving Celebrex has some residual hair, less ulceration and scaring. Animals given EsA had swelling but some preservation of hair and significant reduction in ulceration.



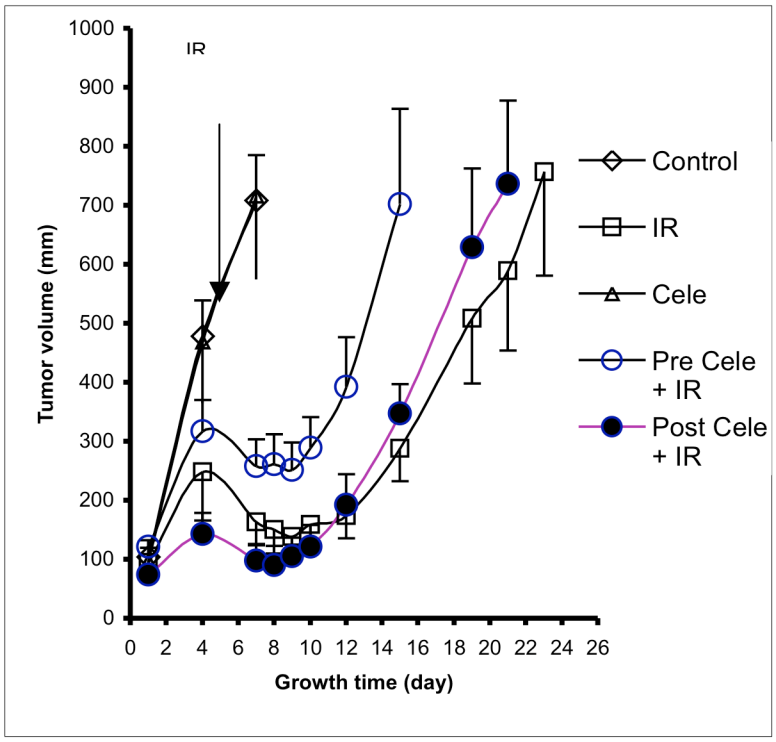


Figure 7. Tumor growth curves for control, Celebrex (Cele), irradiation (IR) and combined treatment are shown. Independent of the timing of celebrex delivery,

before or after irradiation, there was an increase in tumor response. Tumors were the murine MCa35. Radiation dose was 10 Gy. Radiation alone produced the expected 7-8 day growth delay. Celebrex added an additional 2 to 3 day growth delay indicating that the skin protection was complemented by a concurrent tumor sensitization. Importantly the was no evidence of tumor radioprotection by Celecoxib. Confirming the role of Celecoxib is related to a direct tumor effect rather than an interactive radiosensitization, Celecoxib was just as effective given after irradiation as it was given before irradiation.

KEY RESEARCH ACCOMPLISHMENTS:

While we had imperfect ability to perform the experiments exactly as original defined due to unexpected technical difficulty, we succeed in testing all the hypotheses defined in the grant application including mechanistic studies of IL-1 related signal transmission. We believe the results will precipitate a paradigm shift in scientific approach to radiation protection. We have shown that cutaneous side effects of radiation to the skin are not unavoidable or irreversible. Perhaps most importantly, the deviations from the original study design created better experiments and helped advance the translation of our findings to the clinic. In particular we have now identified at least 3 agents that might be used in clinical trials. One such trial is now at CTEP where it has been approved for human testing in women undergoing breast irradiation, pending an IND from the FDA. A copy of that protocol is available on request.

REPORTABLE OUTCOMES:

See appendix for a paper in submission.

CONCLUSIONS:

IL-1 and TGFbeta1 are associated with increased radiation fibrovascular complications. Modifying their expression powerfully modifies both acute radiation dermatitis and late cutaneous fibrosis. Inhibition of these agents specifically or non-specifically in murine mammary cancer tumors actually improves the tumor response. The therapeutic gain of these agents is thus substantial and deserves clinical trials.

REFERENCES:

None sited.

Appendix

IL-1b Signaling is a Critical Component of Radiation-Induced Skin Fibrosis

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Revised Manuscript (6672-L)

IL-1 Signaling is a Critical Component of Radiation-Induced Skin Fibrosis

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Revised Manuscript (6672-L)

Running head: IL-1 and MMPs in radiation-induced skin fibrosis

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ABSTRACT

Interleukin-1 beta (IL-1), a potent pro-inflammatory cytokine, is directly up-regulated by radiation and is known to regulate other inflammation-related molecules, such as the matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs). However, the interaction of IL-1 with MMP and TIMPs in radiation-induced skin fibrosis is unknown. We examined the response of primary dermal keratinocytes, fibroblasts, and endothelial cells to single fraction radiation (10 Gy) and compared the results to a temporal sequence of histology from irradiated C57BL/6 and IL-1R1 knockout mice. These studies showed that keratinocytes are the major IL-1 producing cells in vitro, and that radiation induces an immediate and chronic elevation in the expression of IL-1 mRNA in the skin of C57BL/6 mice. This elevation was principally early and was less pronounced in the IL-1R1 knockout strain, which also demonstrated reduced late radiation fibrosis. Radiation also increased expression of MMP mRNA expression in C57BL/6 mice. Finally, exogenous IL-1 protein induced robust endogenous IL-1 mRNA expression, along with a brisk increase in MMPs and collagen III, but only in the C57BL/6 mice. In conclusion, these data suggest that IL-1 plays a critical role in radiation-induced fibrosis and that the increased MMPs fail to block the IL-1-related collagen accumulation.

Keywords: radiation, fibrosis, IL-1 , IL-1Ra, IL-1R1, MMP, collagen.

INTRODUCTION

Early radio-dermatitis and late radiation fibrosis are consequences of radiation therapy that can produce non-healing wounds, pain, and functional problems (*1-4*). To date, the only proven approaches for reducing radiation toxicity are to decrease the treatment field size, fraction size, or total dose (*5*), or to utilize antioxidants (*6-7*). Although more recent radiation therapy schedules have reduced the levels of skin toxicity, the heterogeneous nature of patient sensitivity has meant that the development of late fibrosis cannot always be avoided (*8-9*). Recent studies indicate that the direct effects of radiation alone are not completely responsible for toxicity, and that inflammatory and fibrogenic cytokines, particularly TGF 1, play an important role in the pathogenesis of fibrovascular toxicity (*10-12*). Elucidation of the mechanisms underlying the development of chronic skin effects should allow for the prediction of patients at risk. More importantly, a better understanding could identify targets for therapies leading to the prevention and mitigation of toxicity.

The pathologic progression of radiation toxicity in many normal tissues appears to feature an inflammatory phase followed by late stromal alterations: for example, pneumonitis followed by pulmonary fibrosis, radiation nephritis followed by renal sclerosis, etc. (*13*). This is also seen in skin. We therefore have hypothesized that inflammatory cytokines play a critical role in the events leading to radio-dermatitis and dermal fibrosis. In particular, our studies have focused on the role played by the interleukin-1 (IL-1) family of pro-inflammatory cytokines. The IL-1 family of cytokines has a large number of biological functions that, with relevance to dermal fibrosis, include enhancing proliferation of keratinocytes and fibroblasts, induction of synthesis of matrix proteins, and possibly increasing collagen production (*14-20*). In addition, it has been suggested that IL-1 may regulate the balance between matrix deposition and degradation (*17-22*).

Specifically, while collagen accumulation in tissue can decrease when tissues are exposed to IL-1 or IL-1 as measured by radioimmunoassay, procollagen mRNA at the pre-translational level increases. TNF appears to be among the signal molecules that powerfully regulate the profibrotic action of IL-1 (*17, 18*). IL-1 is one of the few cytokines shown to be directly induced by radiation, and our group recently presented data demonstrating that radiation significantly induced IL-1 in mouse skin (*23*). We also found that production of IL-1 correlated with radiation-induced cutaneous toxicity on a mouse by mouse basis.

As well as being a potent pro-inflammatory cytokine, IL-1 is involved in the regulation of other inflammation- and fibrosis-related molecules, including the matrix metalloproteinases (MMPs) (*22, 24-26*). MMPs are a group of zinc-dependent enzymes that regulate or degrade protein components of the extracellular matrix (*27, 28*). Their activity is regulated through a counter-balancing system including tissue inhibitors of MMPs (TIMPs). Altered MMP regulation has been associated with inflammation, cell proliferation, cell death, and tissue remodeling in a number of different tissues (*29, 30*). The MMP/TIMP system also plays a significant role in several pathological conditions, including tumor metastasis, angiogenesis, and, importantly, fibrosis (*31-33*). A number of investigators have established a relationship between IL-1 and specific MMPs, although the data have been inconsistent and complex (*16-20, 25, 26, 34*). With respect to the present study, the roles of either IL-1 or MMP/TIMP in radiation-induced skin late effects are unknown. Therefore, the goals of these current studies were to better understand the potential contribution of IL-1 expression in radiation-induced skin fibrosis by identifying: (1) which cell types in cutaneous tissue are responsible for IL-1 production; (2) determining whether IL-1 elevation correlates with MMP, TIMP, and collagen induction; and

(3) in order to establish a causal relationship, evaluating the effects of exogenous recombinant IL-1 on downstream expression of both interleukins and MMP/TIMP.

MATERIALS AND METHODS

Irradiation

Both *in vivo* and *in vitro* irradiations were performed on a J. L. Shepherd Irradiator housing a 6000 Ci ^{137}Cs source. Animals were restrained in custom-made jigs and radiation was applied locally to the right hind limb. The doses were administered as described and were delivered at a dose rate of 196 cGy/min.

In vitro Studies

Primary cultured human keratinocytes, fibroblasts, and endothelial cells from the skin of a single donor were purchased (Clonetics). Dermal fibroblasts were grown in fibroblast cell basal medium (modified MCDB202) (Clonetics) supplemented with 0.5 µg hFGF-B, 2.5 mg insulin, 0.5 ml GA-1000, and 10 ml FBS in 500 ml of medium. Dermal keratinocytes were grown in keratinocyte basal medium (modified MCDB153) (Clonetics) with the following growth supplements: 15 mg bovine pituitary extract, 0.05 µg hEGF, 2.5 mg insulin, 0.25 mg hydrocortisone, and 0.5 ml GA-1000 in 500 ml of medium. Dermal endothelial cells were grown in endothelial cell basal medium-2 (CCMD130) (Clonetics) with 5% FBS, 5µg hEGF, 0.2 mg hydrocortisone, 0.5ml GA-1000, 0.5 ml VEGF, 2 ml hFGF-B, 0.5 ml R_3-IGF-1, and 0.5 ml ascorbic acid in 500 ml of medium. The cells were cultured at 37 C in a 5% CO_2 atmosphere in 150 cm^2 tissue culture dishes. After reaching 75-80% confluence, cells were irradiated with a

single dose of 10 Gy and harvested with TRI Reagent (MRC) at 0, 2, 8, 12, and 24 h after irradiation. Only early passage cells (up to six or seventh passage) were used in the experiments.

Animals

C57BL/6 mice were purchased from the National Cancer Institute. Breeding pairs of mice with a homozygous knockout for IL-1R1 (hereafter designated IL-1R1-/-) were purchased from Jackson Laboratory and were heavily back-crossed into the C57BL/6 line. Equal numbers of male and female mice, 8-12 weeks old, were used. IL-1R1 knockout mice have a nearly indistinguishable phenotype from wild type and have normal life expectancy. They therefore make an excellent model for studying the role of IL-1 signal after irradiation. Other models such as the IL-1Ra knockout mouse (*35*) develop severe spontaneous inflammatory conditions, which makes its use in long-term studies evaluating the inflammatory effects of radiation problematic. Other mouse models, including animals that over-produce IL-1 in the basal keratinocytes (*36*), also develop spontaneous inflammatory toxicity, and were therefore not used. C57BL/6 (n=70) or IL-1R1 (n=70) mice were divided into 3 groups [non-radiation control (n=30), 30 Gy (n=33), 30 Gy and recombinant IL-1 (30 Gy+rIL-1)(n=7)]. The mice were sacrificed with CO_2 and the skin harvested on days 19 (30 Gy n=9), 60 [(30 Gy n=15) and (30 Gy+rIL-1 n=7)], and 90 (30 Gy n=9) post-radiation. Mice were handled in accordance with the standards established by the U.S. Animal Welfare Acts set forth in the National Institutes of Health (NIH) guidelines. All procedures were reviewed and approved by the University of Rochester's Committee on Animal Resources.

Recombinant IL-1 Administration

IL-1 recombinant protein (Upstate Biotechnology) was injected subcutaneously into the irradiated mouse limbs (n=7) on days 0, 3, 6, 9, and 12 beginning immediately after irradiation (one 10 ng dose every 72 hr for a total of 50 ng per mouse). Five saline injections were administered at the same intervals into control animals [negative (0 Gy n=3) and positive controls (30 Gy n=6)]. Animals were sacrificed and the skin harvested on day 60 post-radiation.

ELISA Assay

ELISA was used to measure protein levels of IL-1 in the primary human keratinocyte, fibroblast, and endothelial cell cultures. Cell lysate was collected using lysis buffer as previously described (*37*). The ELISA for human IL-1 (R&D System) was performed according to the manufacturer's instruction. A standard curve with cytokine-positive control was run in each assay, and the lower limit of detection was determined to be 2 pg/ml for IL-1 .

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) to analyze the induction of cytokines including IL-1 , IL-1 ,and IL-1R1 mRNA from the skin of C57BL/6 and IL-1R1-/- mice. Briefly, for cDNA synthesis, 1 µg of total RNA was transcribed with TaqMan reverse transcription reagents using random hexamers. The manufacturer's instructions were used with minor changes. Primers and TaqMan probes were designed using the primer design software Primer Express (Applied Biosystems). The GAPDH was available commercially. The IL-1 was forward primer (5'-

CACTATCTCAGCACCACTTG), and reverse primer (5'-CTGGAAGTCCATGAGGC). The IL-1 primer was as follow: forward primer (5'-AACCTGCTGGTGTGTGACGTTC), reverse primer (5'-CAGCACGAGGCTTTTTTGTTGT). The IL-1R1 was forward primer (5'-GGTGACAGTAACTGGTGTT) and reverse primer (5'-ACGGTGGGGAAGACATTGTT). The cDNA sample (100 ng) was mixed with primers and TaqMan Universal PCR Master Mix in a total volume of 50 µl as described in the manufacturer's directions (protocol 4304449; Applied Biosystems). The PCR was conducted using the following parameters: 50 C for 2 min, 95 C for 10 min, and 40 cycles at 95 C for 15 s and 60 C for 1 min. Quantitative real-time PCR was performed for IL-1 , IL-1 , IL-1R1, and GAPDH in triplicate. The fold change of IL-1 , IL-1 , and IL-1R1 mRNA were normalized to the copies of GAPDH mRNA from the same sample. Acquired data were analyzed by sequence detection software version SDS 2.1 (Applied Biosystems). Tissue from each individual animal (n=9) was measured independently to determine average and standard error.

RNase Protection Assay

The total RNA from each mouse skin sample or each cell type was prepared and extracted for RNase protection assay according to the manufacturer's protocol (PharMingen). RNase protection was performed using established multiprobe template sets (PharMingen), as previously described (*38*). Briefly, 20 µg of total RNA from cells or skin was hybridized to a ^{32}P-labeled CK2 or MMP template set (6 x 10^5 cpm per sample) for 16 h at 56 C, and then subjected to RNase A+T1 digestion, phenol chloroform extraction, and ethanol precipitation. The samples were run on a 7% denaturing polyacrylamide gel (National Diagnostics). Human or mouse CK2 and mouse MMP templates were used for assays, as appropriate. The components of the mouse

CK2 multiple template set are IL-12p35, IL-12p40, IL-10, IL-1 , IL-1 , IL-1Ra, IL-18, IL-6, IFN , MIF, L32, and GAPDH. The human CK2 set is similar but does not include MIF. For MMPs and TIMPs, a RiboQuant Custom Mouse Template Set was used and the multiple template included MMP-1/13, -2, -3, -7, -8, and -9, and TIMP-1, -2, -3, and -4, as well as L32 and GAPDH. The quantification of mRNA expression level for each sample was measured using a Cyclone Phosphorimager (HP Company). Relative mRNA expression level was measured as the ratio of the targeted gene divided by the internal control, L32.

Western Blot Assay

Protein levels of IL-1 , IL-1 ,IL-1Ra, and IL-1R1 in the skin of irradiated and non-irradiated mice were determined by Western Blot assay. Protein acquired from skin of the irradiated leg was lysed in buffer (M-PER, Mammalian Protein Extraction Reagent). A total of 50 µg of protein was loaded into each lane on the SDS-PAGE polyacrylamide gels and electro-transferred onto polyvinyl difluoride membrane (NEN). The blots were incubated overnight at 4°C with 1:1000 IL-1 , IL-1 ,IL-1Ra, and IL-1R1 polyclonal antibodies (Santa Cruz). Thereafter, membranes were washed in Tris-buffered saline and incubated with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (1:2000) for 1 h. The blots were visualized by chemiluminescence using the ECL Plus (Amersham Corp.).

Histology

At time points 0, 19, 60, and 90 days after irradiation, the animals were sacrificed and their limbs removed and processed. Tissue for histological evaluation was fixed in formaldehyde and embedded in paraffin. Five-micron sections were cut and stained with hematoxylin and eosin by

conventional methods. Immunohistochemical staining for type III collagen (S-17, goat polyclonal IgG, epitope mapping at the N-terminus) and IL-1 (C-20, goat polyclonal IgG, epitope mapping at the C-terminus) were performed according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Negative sample control was employed and specimens from different time points were stained in batches to allow useful intercomparisons.

RESULTS

Evaluation of the Histopathological Progression of Fibrosis

The histopathological progression of the dermal response to a single dose of 30 Gy is illustrated in Figure 1. Normal non-irradiated skin is similar in both C57BL/6 and IL-1R1-/- mouse strains (left column). Specifically, they both had a thin layer of epidermis, a sub-epidermal papillary dermal layer, and a dermis with a delicate network of fibrous stroma and fat. By 19 days post-radiation, C57BL/6 mice developed an inflammatory reaction in the papillary dermis that spread up to the epidermis, and included matrix deposition and atrophy of the papilla. Peak early desquamation occurred at 19 days and is severe in the C57BL/6 mice. At later stages (days 60 and 90 post-radiation), fibrosis progressed to include the deeper dermis and includes hair loss and dermal hyperplasia. These late changes were far less pronounced in the IL-1R1-/- mice.

IL-1 over-expression is restricted to the epidermis in vivo.

In Figure 2, IL-1 immunohistochemical staining shows that IL-1 over-expression is restricted to the epidermis. IL-1 staining is increased at all time points including early radiation dermatitis (day 19) and late radiation fibrosis (day 60 and 90) in the C57BL/6 mice.

Effect of Radiation on Primary Keratinocytes, Fibroblasts and Vascular Endothelial Cells

To gain an understanding of the cell or cell types from which the IL-1 signal originates, we studied early generation normal tissue cell lines developed from a single human donor. Figure 3 shows mRNA and protein expression from keratinocytes, fibroblasts, and dermal vascular endothelial cells. The gel in Figure 3A is representative of experiments performed in duplicate measured by RNase protection assay. As can be seen, there was a significant induction of mRNA expression of IL-1 , IL-1 , and IL-1Ra in the keratinocytes (Fig. 3B). This dramatic elevation in IL-1-related signal proteins in keratinocytes was immediate and prolonged. Of note, other inflammatory cytokines in the same probe set, including IL-6, were not induced in any of the cell lines following irradiation, indicating that the effect on the IL-1 signal pathway is relatively specific and not part of a general inflammatory reaction. Cell lysate collected at different time-points after irradiation were also analyzed for the levels of IL-1 protein using ELISA. Protein production was increased at 2 hours post-radiation, and, again, the highest levels were seen in the keratinocytes (Fig. 3C).

Analysis of interleukin mRNA expression and Protein Production in the Skin of C57BL/6 and IL-1R1 -/- Mice

Following single dose irradiation with 30 Gy, measurement of the total skin RNA was made from the right hind legs of C57BL/6 and IL-1R1-/- mice on days 0, 19, 60, and 90. Quantitative real-time PCR was performed in triplicate for IL-1 , IL-1 , IL-1R1, and GAPDH. Individual animals were assayed separately for statistical analysis, and combined for an *n* of 9 assays per group; results from the analysis using the $2^{-\ \ C_T}$ method are shown in Table 1 (*39*). As can be seen, skin levels of IL-1 mRNA were significantly elevated in both C57BL/6 ($P < 0.01$) and IL-

1R1-/- ($P < 0.05$) mice at 19 days post-radiation. As indicated in Table 1, C57BL/6 mice had a self-amplified response (75-fold) compared with IL-1R1-/- mice (7-fold), since the latter are genetically unable to respond to the radiation-induced cytokine. Similarly, on days 60 and 90 post-radiation, the IL-1 mRNA expression was only elevated in the C57BL/6 mice. The C57BL/6 animals also demonstrated small increases in the relative levels of IL-1 mRNA, which were not seen in the IL-1R1-/- mice. IL-1R1 mRNA expression was increased on days 19 and 60 post-radiation in the C57BL/6 mice. Of course no IL-1R1 response was seen in the IL-1R1-/- mice. Figure 4 illustrates the IL-1 protein expression as measured by western blot. IL-1 increased immediately after irradiation and remained chronically elevated, especially in the C57BL/6 mice. IL-1 increased slowly and gradually with time after irradiation in both C57BL/6 and IL-1R1-/- mice, but never reached the level of IL-1 . In contrast, IL-1Ra protein expression appeared to be suppressed during the time phase that fibrosis progresses in the C57BL/6 animals. The IL-1Ra protein expression appears complex in the IL-1R1-/- mice, but again it must be noted that IL-1Ra has no target receptor in these animals.

Analysis of MMP and TIMP Expression Following Irradiation

To investigate for correlation between IL-1 signal and MMP/TIMPs, single hind limbs of C57BL/6 and IL-1R1-/- mice received 30 Gy irradiation. Total skin RNA was analyzed by RNase protection assay for mRNA expression of MMPs and TIMPs at days 19 and 90 post-radiation; these time points represent early dermatitis and late fibrosis, respectively. Individual animals were assayed separately for statistical analysis and comprised an *n* of 4-6 animals per group. Substantial variability was observed between animals. In C57BL/6 mice, at both time points, the relative mRNA levels of MMP-1/13, -2 and -3 were higher than normal skin (Figure

5A), but the effect was only significant on day 19 for levels of MMP-2 ($P < 0.05$) and MMP-3 ($P < 0.01$). There was no change in MMP levels in the IL-1R1-/- mice and no alteration in levels of expression of TIMPs following radiation in either strain of mouse (Figure 5B).

Effects of Endogenous Recombinant IL-1 in Irradiated Mouse Skin

The effects of the administration of recombinant IL-1 were evaluated by RNase protection assay in C57BL/6 and IL-1R1-/- mouse skin at 60 days post-radiation (30 Gy) (Figure 6). In C57BL/6 mice, the exposure to exogenous IL-1 during the first 2 weeks induced robust expression of endogenous IL-1 (Figure 6A) on day 60. This was not seen in the IL-1R1-/- animals. This is consistent with the apparent self-amplification of IL-1 seen in the C57BL/6 animals in Table 1. Recombinant IL-1 also caused a significant increase in expression of MMP-1/13, -2 and -3 (Figure 6A) that was not seen in the IL-1R1-/- mice (Figure 6B).

Changes in Type III Collagen in Irradiated Mouse Skin

Figure 5 demonstrated that MMP-3 was the most responsive protease at the early (19 day) time point. Collagen III, one of the more abundant collagens in cutaneous fibrosis (*40*), is a substrate for MMP and TIMP activity. We therefore analyzed collagen III levels in order to establish any correlations between collagen and MMP/TIMP activity (Figure 7). Immunohistochemistry demonstrated heavy positive cytoplasmic staining for type III collagen at 60 and 90 days after radiation in irradiated skin of C57BL/6 mice. There were no significant changes in the level of collagen III staining during early radio-dermatitis in either strain (day 19); and no increases were observed in IL-1R1-/- mice at any time point.

DISCUSSION

In the present study, we examined the role of IL-1 signaling in radiation-induced cutaneous fibrosis. We observed a histopathological progression of fibrosis showing that loss of the IL-1R1 receptor led to a modification in skin response (Figure 1). The IL-1R1-/- mice had mild histopathological reactions at all observed time points, little desquamation at early time points, and reduced hyperkeratosis at late points. These data from the knockout strain suggest a key role for IL-1 in dermal fibrosis development. This was supported by the results from the C57BL/6 animals. Specifically, the early reaction, seen at day 19, was predominantly sub-epidermal, consistent with the *in vitro* data indicating that keratinocytes are a critical source of IL-1 (Figure 4). Thus, IL-1 produced by the keratinocytes diffuses to the immediately adjacent dermis causing reaction by paracrine effect. Also consistent with keratinocytes predominantly accounting for post-radiation IL-1 production, the epidermis was the single tissue compartment with substantially increased IL-1 protein (Figure 2). IL-1 and/or its receptors can be expressed by fibroblasts and endothelium; however, based on our data, their impact on radiation response appears to be of secondary importance.

The hyperkeratosis that occurs after irradiation is less studied than subcutaneous fibrosis. Transgenic mice that over-express IL-1 in the basal keratinocytes develop hair loss, scaling, and hyperkeratosis with a monocytic infiltrate very similar to that seen in our animals after irradiation. Cutaneous IL-1 production therefore can account for the hyperkeratosis and other skin changes we observed. Other chronic inflammatory stimuli, including those due entirely to cytokines like IL-4, interferon, KGF, and IL-12 can also cause hyperkeratosis (*36, 41*).

Evidence that IL-1 stimulates MMP expression has previously been reported *in vitro* (*42, 43*). Whether IL-1 promotes further IL-1 expression through an autocrine loop and can

affect MMP expression levels *in vivo* is unknown. We demonstrated that delivery of exogenous recombinant IL-1 to supplement the naturally occurring response to radiation not only caused an increase in downstream IL-1 production, but also increased MMP production. These increases were prolonged, continuing weeks after the stimulatory period. This observation supports our belief that prolonged exposure to IL-1 chronically upregulates itself and provides a path for chronic overexpression of IL-1. We also showed that the chronically high IL-1 levels were associated *in vivo* with late skin changes and also with increased levels of MMPs, but not TIMPs. No studies have yet focused on other modifiers of stromal architecture, particularly those that modify extracellular matrix such as matrilysin. Data support our hypothesis that radiation-induced IL-1 signaling, rather than TIMP activity, plays a role in early dermatitis and late radiation fibrosis; however, they do not completely explain why fibrosis occurs and suggest that there are other as yet unknown factors involved.

Since IL-1 is believed to modulate MMP mRNA *in vivo*, it is inferred that these MMPs play a role in fibrosing inflammatory disease conditions, including cutaneous keloids (*10*) and lung fibrosis (*44, 45*). Thus, we hypothesize that there is a similar IL-1/MMP relationship as an inherent component of radiation-induced dermal fibrosis, occurring through the following mechanism. Immediately after irradiation, keratinocytes express increased levels of IL-1 (Figure 2) and this expression leads to macrophage infiltration and activation (supported by data in Figure 1). The activated macrophage produces IL-1 and IL-1 , causing further macrophage infiltration, leading to an even higher IL-1 signal (supported by data in Figure 4). In turn, the tissue responds by remodeling, a process that includes increased production of collagen. MMP increases do overcome collagen deposition during the early reaction (*46, 47*) but cannot

overcome the inflammatory response and resulting collagen accumulation over long time periods (Figure 5). All of these effects are blunted or absent in the IL-1R1-/- mice.

MMPs are collectively capable of degrading essentially all components of the extracellular matrix. They are synthesized and secreted by multiple cell types, including epidermal keratinocytes and dermal fibroblasts in skin (*48, 49*). They are classified into five groups on the basis of substrate specificity: gelatinases, collagenases, stromelysins, membrane-type MMPs, and others (*21, 22*). MMP-1 (collagenase-1), MMP-13 (collagenase-3), MMP-2 (gelatinases A), and MMP-3 (stromelysin-1) degrade or cleave collagens in different tissues (*50*). Our data showed that type III collagen was increased in C57BL/6 dermis at 60 and 90 days following radiation. The collagen deposition was seen in the presence of a chronically maintained increase in IL-1 expression and despite overexpression of several MMP mRNAs, including MMP-3 and MMP-2 mRNA. The epidermal but not stromal type III collagen was seen during early radiation dermatitis (day 19), perhaps because MMP is elevated. In contrast, the type III collagen in IL-1R1-/- mice never increased substantially and there was no downstream MMP response. Based on these data, we concluded that, under normal physiological conditions, the type III collagen production is maintained through a balance of collagen synthesis and MMP-related degradation. During early radiodermatitis (*i.e*. day 19), despite the beginning of a dysregulation, the collagen synthesis/MMP balance still favors a normal collagen distribution. Over time, in the IL-1R1 replete mice, the balance shifts to favor collagen accumulation and fibrosis. It will be interesting in future studies to see how radiation fibrogenesis matures in these animals including some studies of maturation of scarring.

IL-1 signaling is complex. It includes the two forms of IL-1 encoded by distinct genes, IL-1 and IL-1 , and by the main receptor IL1R1. These are opposed by IL-1Ra, which binds

the receptor but does not activate the kinase, and by IL-1R2, which binds IL-1 and IL-1 but does not have kinase activity. The inflammatory activities of IL-1 are partially derived by transcriptionally inducing expression of cytokines such as TNF- and interferons. IL-1R1 also induces the expression of other inflammation-related genes. The signal transduction cascade utilized by IL-1R1 has features similar to that of TNF, including NF-kB activation. Thus inflammation induced by IL-1 signals can be self-amplifying through the TNF also induced by IL-1. Increased IL-1R1 seen at late times following irradiation, can further self-amplify the impact of IL-1 ligand. Finally, the IL-1 pathway can be self-perpetuated by autocrine and paracrine paths. Many cell types, including keratinocytes, macrophages, and fibroblasts, are both induced to proliferate and to express TGFß and/or IL-1 by each other. The TGFß in turn, powerfully stimulates fibroblast maturation and collagen expression, and is known to be critical to radiation- and chemotherapy-induced fibrosis (*11, 51, 52*). We have not yet measured the changes in the many factors that impact the balance of IL-1 signal. In future studies we intend to examine the impact of radiation on IL-1Ra, and on Caspase I, which activates pro-IL-1 for extracellular export.

In conclusion, we have presented evidence indicating that the keratinocyte is a major cell type responsible for the production of IL-1 after irradiation of the skin. The results demonstrate that IL-1 production was amplified by exogenous IL-1 administration and support our hypothesis that the chronic production of IL-1 in irradiated tissues is explained by an autocrine and/or paracrine loop. Finally, it is well known that the balance between collagen synthetase, TIMPs, and MMPs is critical in determining the maintenance of normal collagen structure in skin and other tissues. However, chronically up-regulated IL-1 pathways in skin appear to overcome the ability of MMPs to prevent abnormal collagen deposition. This is supported by our data showing

that IL-1R1-/- mice that are unable to respond to IL-1 have a near absence of radiation fibrosis. Taken together, IL-1 signaling appears to be a critical factor leading to both early and late radiation toxicity of the skin. If this is true, inhibition of IL-1 signal, even after initiation of fibrosis, might lead to remodeling of tissue fibrosis and clinical benefit. We are now beginning studies to further test this hypothesis.

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FIGURE LEGENDS

Figure 1. Histopathological progression of fibrosis following 30 Gy radiation. H&E staining (x 100) illustrates representative skin sections from the hind limbs of C57BL/6 and IL-1R1-/- mice at 0, 19, 60, and 90 days post-radiation. Compared to C57BL/6 animals (top row), IL-1R1-/- mice (bottom row) demonstrated a relatively normal epidermal and papillary epidermal thickness, and hair is present. Though there is mild edema, there is little or no fibrosis.

Figure 2. IL-1 staining in skin after irradiation. Immunohistochemical staining IL-1 after irradiation of C57BL/6 (top row) or IL-1R1 -/- mice (bottom row). IL-1 immunohistochemical staining shows that IL-1 over-expression is restricted to the epidermis *in vivo*. IL-1 staining is increased at all time points including early radiation dermatitis (day 19) and late radiation fibrosis (day 60 and 90) in the C57BL/6 mice.

Figure 3. Effect of 10 Gy radiation on mRNA and protein expression levels of interleukin in primary cultures of keratinocytes, fibroblasts and vascular endothelial cells. The mRNA levels of irradiated cells were measured by RNase protection assay and the ratio to L32 is shown. The mRNA expression level of IL-1 was greatly induced by irradiation in keratinocytes as compared to fibroblasts and vascular endothelial cells (n=2-3) (A) and (B). IL-1 protein levels of irradiated cells were measured using ELISA. Consistent with mRNA expression in (A) & (B) and with the histopathology in Figure 1, the IL-1 protein was most dramatically up-regulated in keratinocytes upon radiation (C). *$P<0.05$.

Figure 4. The effect of 30 Gy radiation on the protein expression of IL-1. The protein expression of IL-1 in the skin of the hind limbs of C57BL/6 versus IL-1R1-/- mice was measured by western blot assay. IL-1ß levels were increased immediately and remained elevated throughout the study. Though the IL-1 related protein production was intact in the IL-1R1-/- mice, the absence of IL-1R1 makes them ineffective.

Figure 5. MMP and TIMP mRNA expression in (A) C57BL/6 and (B) IL-1R1-/- mouse skin after 30 Gy radiation, as measured by RNase protection assay relative to L32. Animals were sacrificed at 0, 19 and 90 days. Significant elevation of MMP-2 and MMP-3 were seen at 19 days post-radiation in the C57BL/6 animals. No change in TIMP expression was seen in either mouse strain at any time point. * = $P < 0.05$; ** = $P < 0.01$, compared with day 0.

Figure 6. Quantification of the fold-changes in mRNA expression of IL-1ß, MMPs and TIMPs relative to L32. Changes in expression were measured 60 days following 30 Gy irradiation. Supplemental administration of recombinant IL-1ß was given as described in the methods. RNase protection assays were used in C57BL/6 (Figure 5A) and IL-1R1-/- (Figure 5B) mice. The early administration of exogenous IL-1ß caused an increase in IL-1ß expression above radiation alone in the C57BL/6 mice and was also associated with increased MMP levels. No increases in expression were seen in the IL-1R1-/- mice. Asterisks represent *P* values for radiation versus controls: * = $P < 0.05$ vs. 0 Gy; †= $P < 0.05$ vs. 30 Gy alone.

Figure 7. Collagen III staining in skin after irradiation. Immunohistochemical staining for type III collagen after irradiation of C57BL/6 (top row) or IL-1R1 -/- mice (bottom row). Collagen III staining is similar in non-irradiated wild type and IL-1R1 -/- mice. After irradiation

collagen III staining is increased at all time points including early radiation dermatitis (day 19) and late radiation fibrosis (day 60 and 90). The increase in stromal staining, however, is far more prominent and progressive in the C57BL/6 mice.

Table 1. RT-PCR Analysis of Skin Following 30 Gy Irradiation
Using the 2^{-C_T} Method (± 1 S.D.)

Time Post-Radiation (days)	Fold Change in IL-1 mRNA Expression (n = 9)					
	IL-1		IL-1ß		IL-1R1	
	C57BL/6	IL-1R1-/-	C57BL/6	IL-1R1-/-	C57BL/6	IL-1R1-/-
0	1	1	1	1	1	nil
	(0.9 – 1.1)	(0.6 – 1.6)	(0.8 – 1.3)	(0.5 – 2)	(0.8 – 3)	
19	2.7*	1.4	75*	7.4*	1.7	nil
	(1.8 – 4)	(0.6 – 3)	(56 – 102)	(2.6 – 21)	(1 – 2.9)	
60	3.3*	1.1	25*	1.1	3.6*	nil
	(2.6 – 4.3)	(0.6 – 2.2)	(4 – 167)	(0.4 – 2.8)	(3.1 – 4.2)	
90	1.3	1.4	9.2*	1.6	0.9	nil
	(0.6 – 2.9)	(0.6 – 3.3)	(2.4 – 35)	(0.4 – 6.6)	(0.3 – 2.3)	

* $P < 0.05$

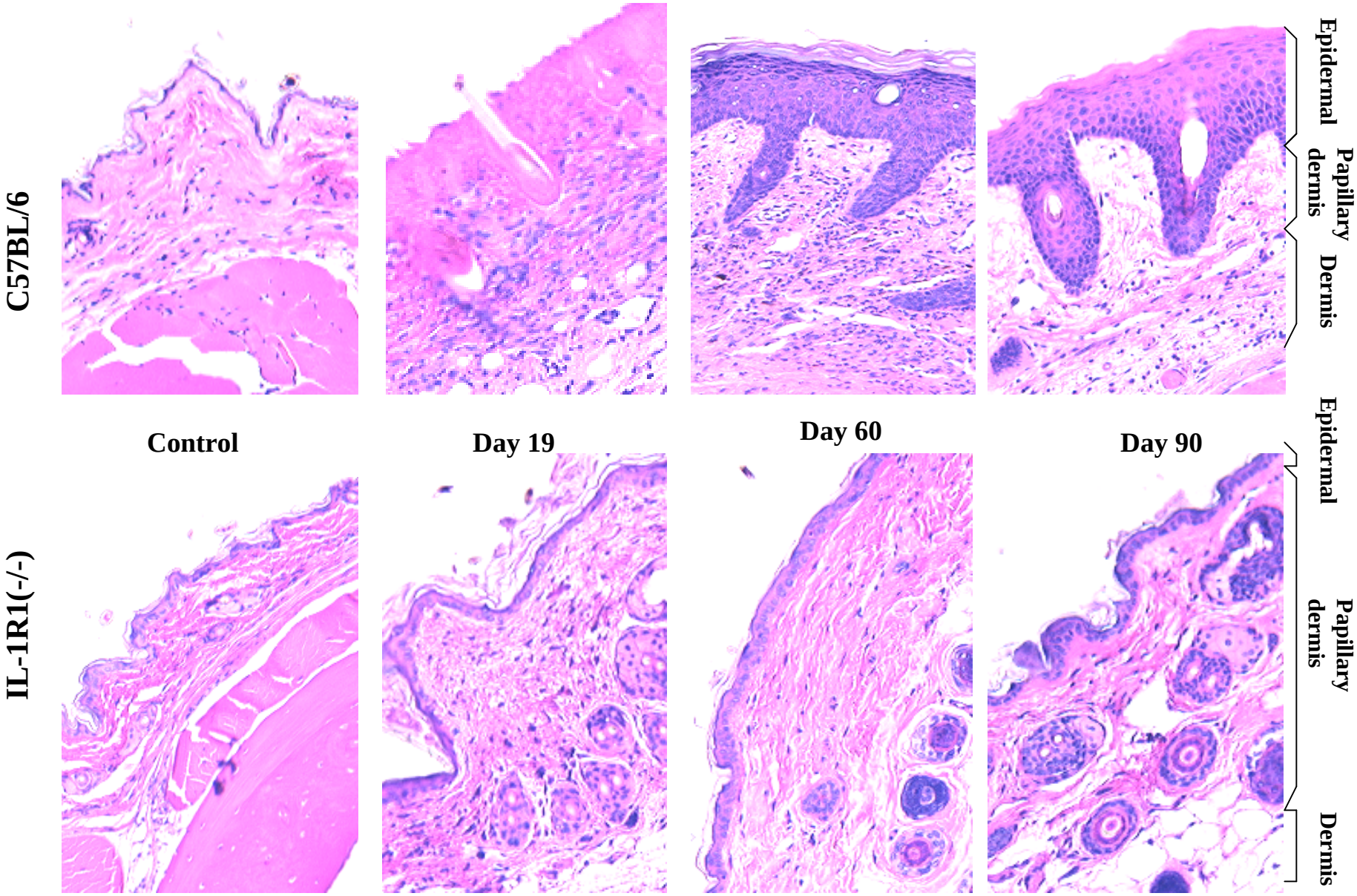


Fig. 1. Liu et al.

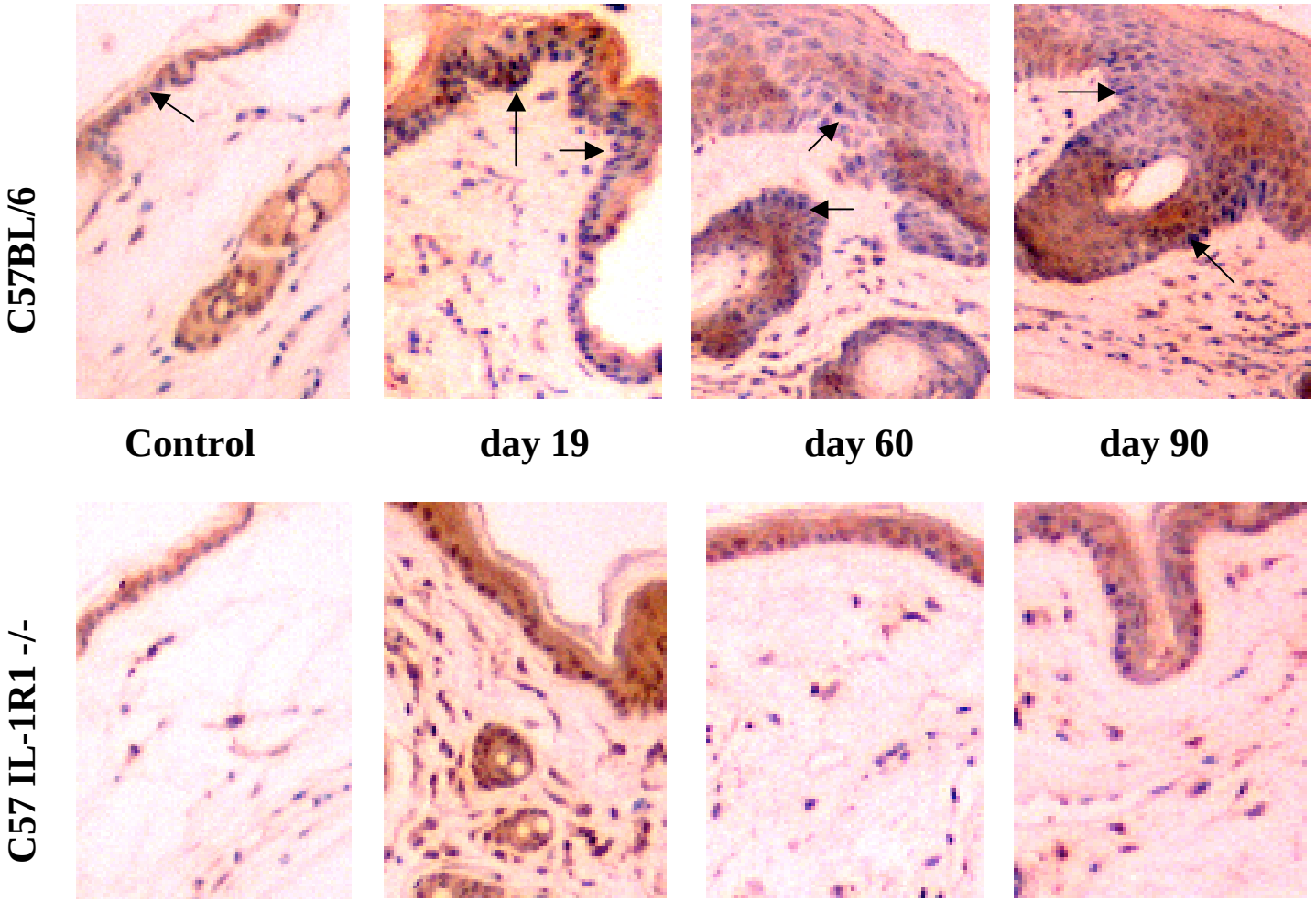


Fig. 2. Liu et al.

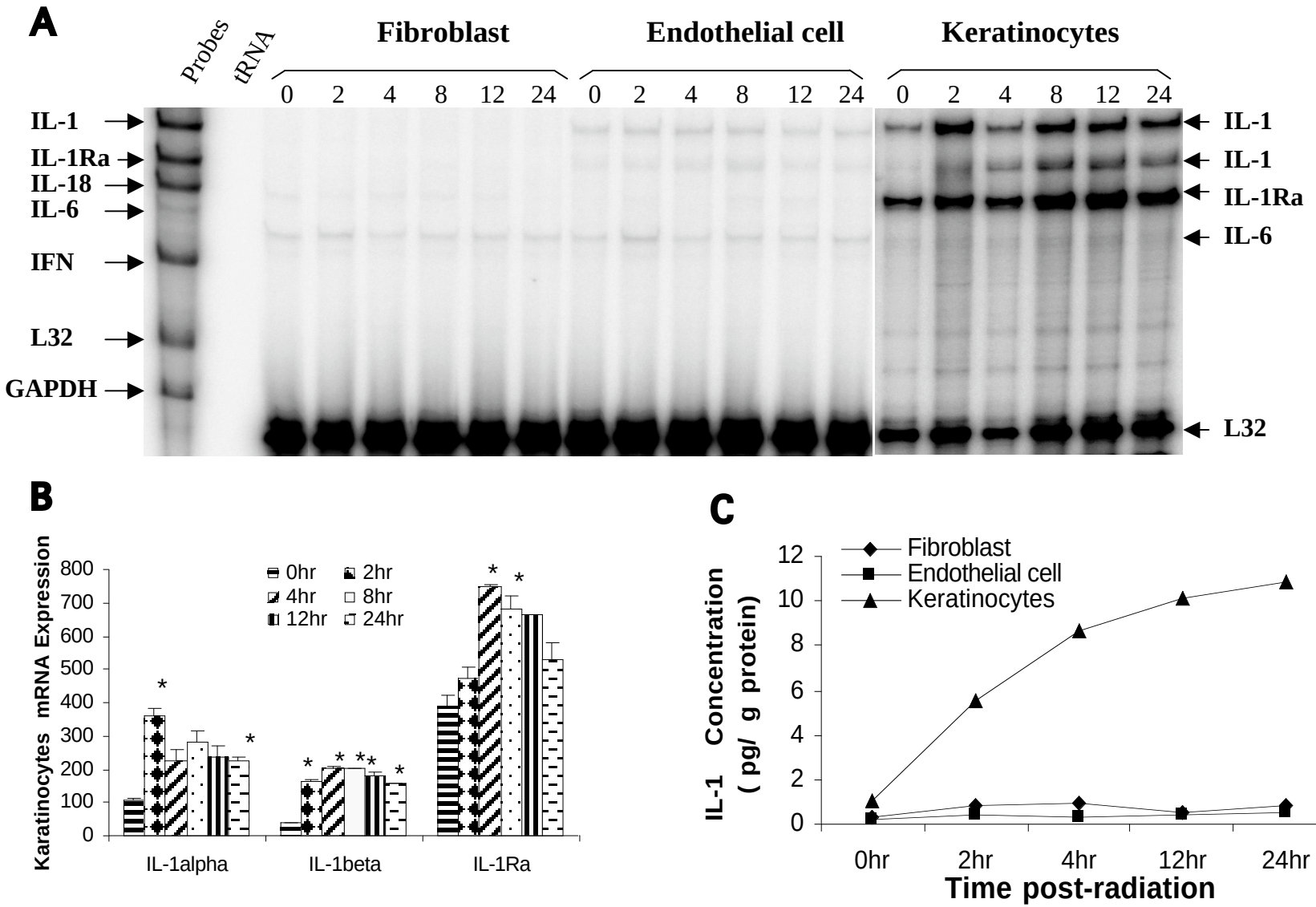
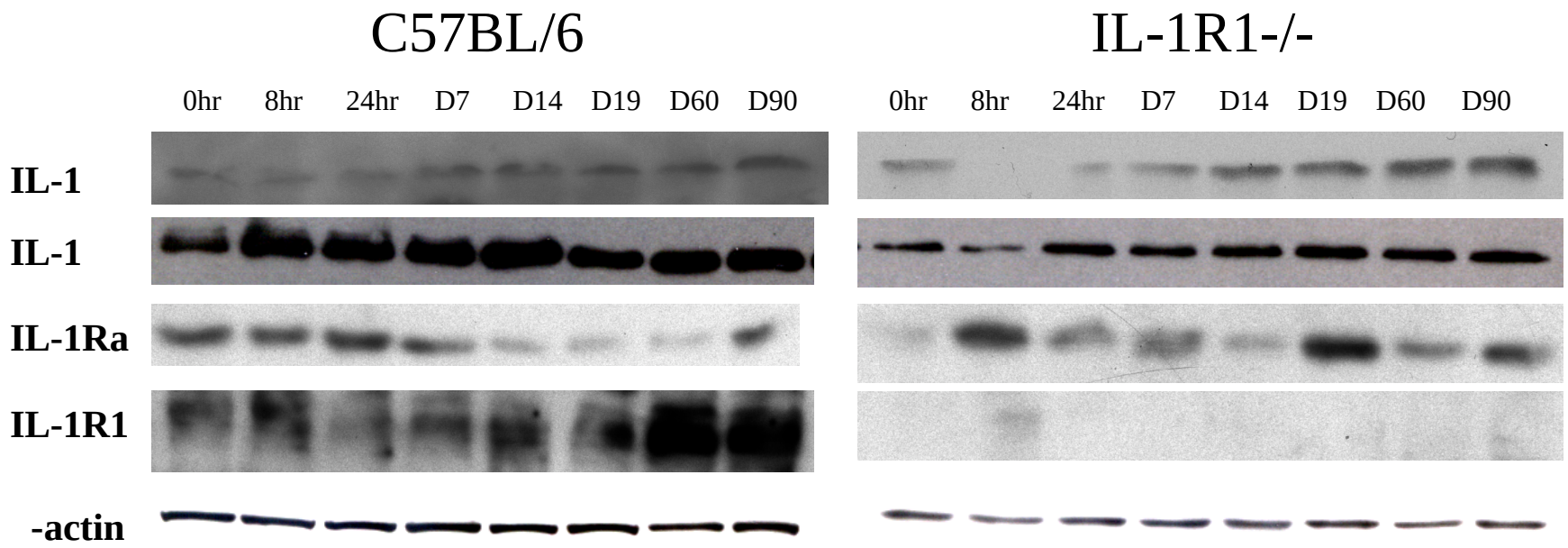


Fig. 3. Liu et al.



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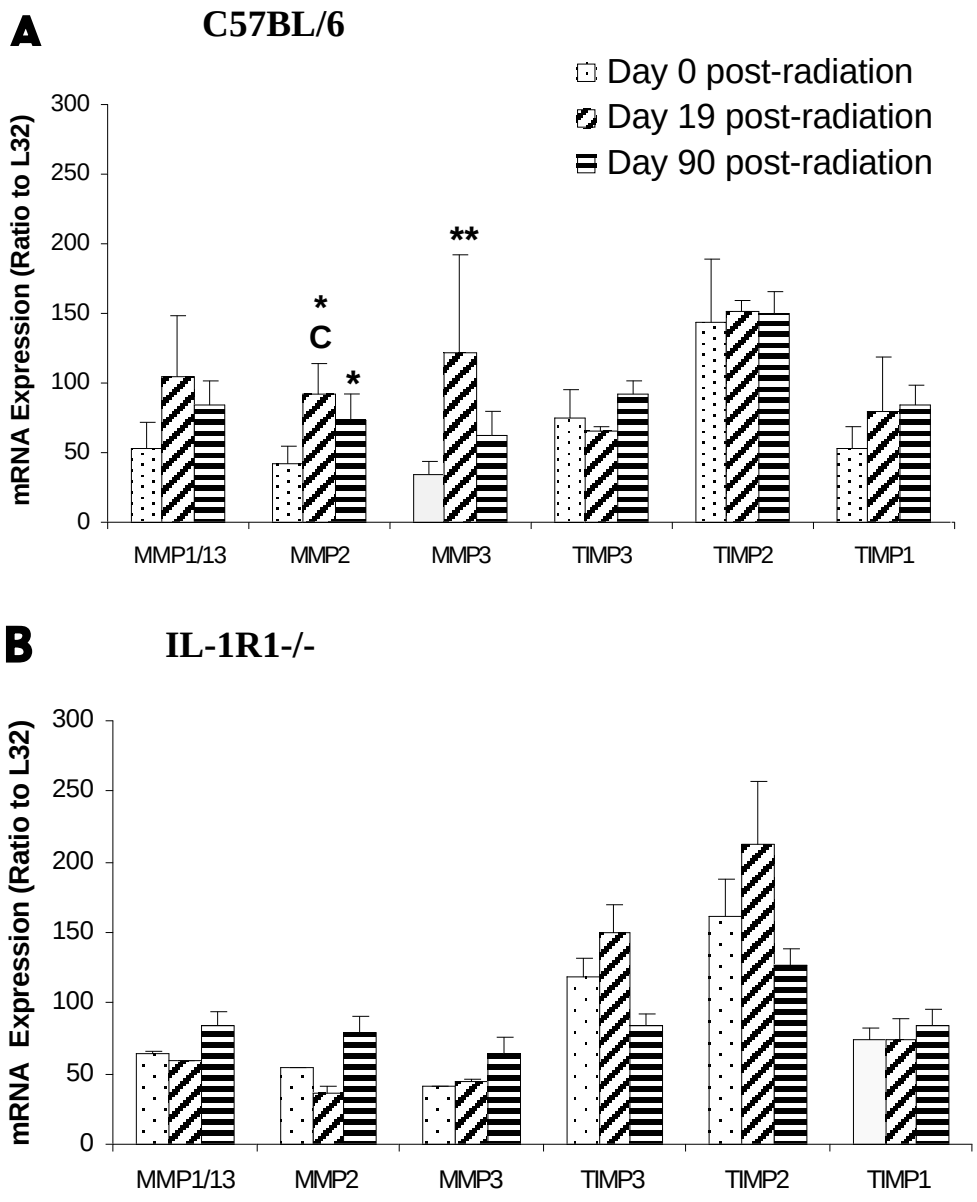

Fig. 5. Liu et al.

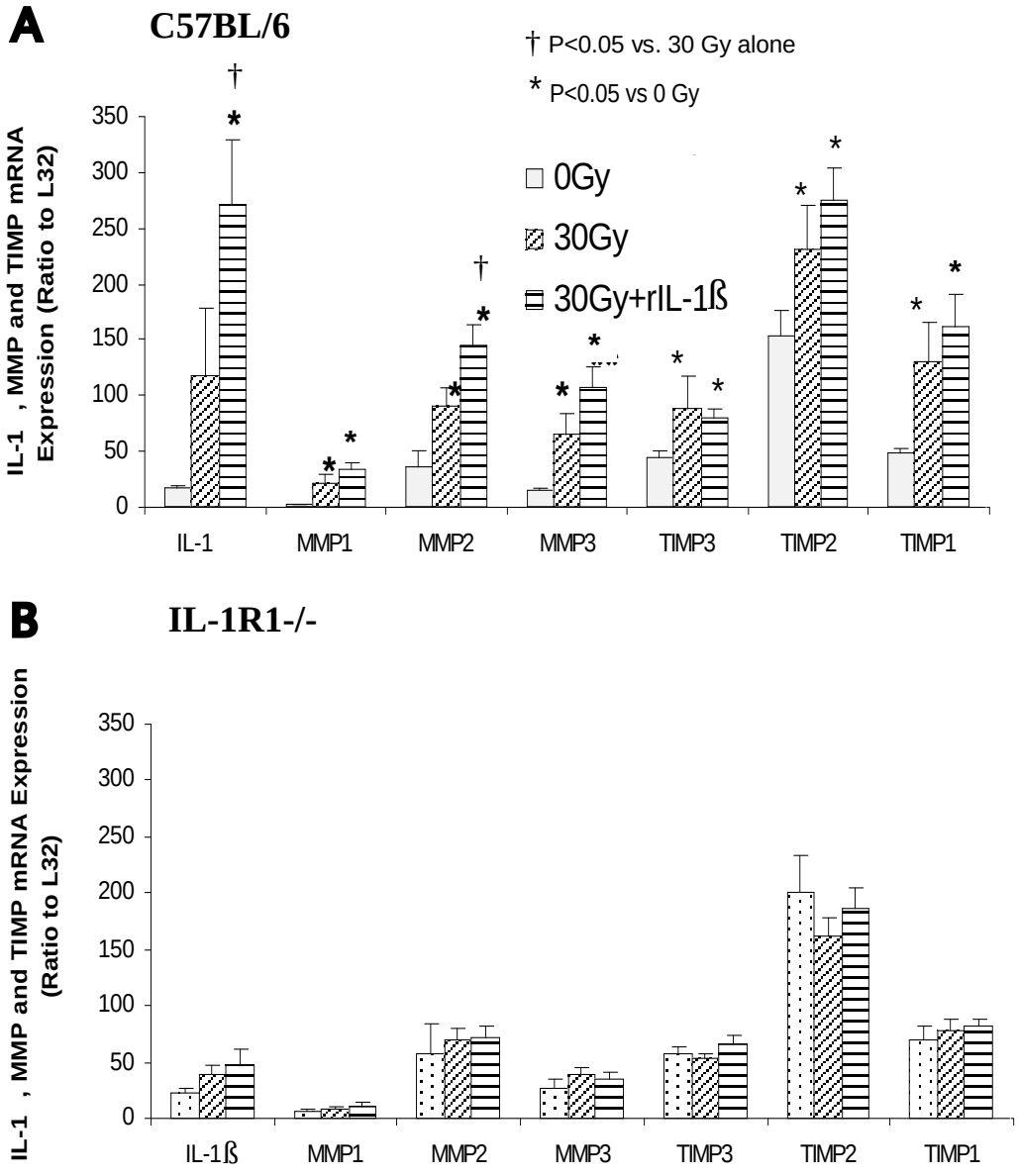

Fig. 6. Liu et al.

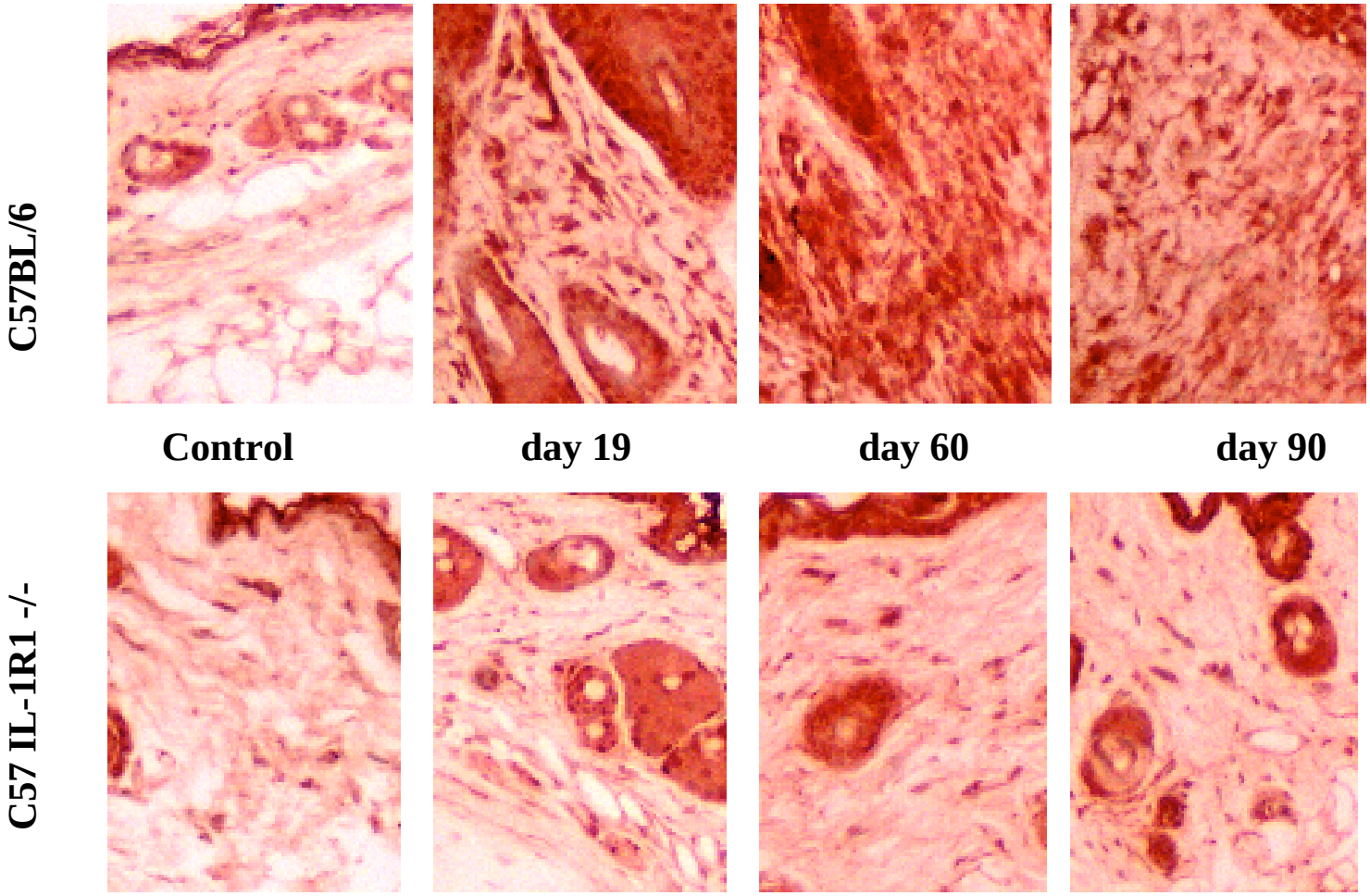

Fig. 7. Liu et al.